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TITLE: Inhibition of the Protein Tyrosine Phosphatase, SHP-1, in Dendritic Cells to Enhance their Efficacy as Cell-Based Prostate Cancer Vaccines

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14. ABSTRACT
Early preclinical and clinical trials suggest that dendritic cell (DC)-based tumor vaccines are both feasible andsafe. However, to date clinical trials of DC-based vaccines have demonstrated only limited efficacy in causing tumorregression despite eliciting measurable systemic T cell responses against prostate cancer. In an effort to enhancethe effectiveness of DC-based vaccines against prostate cancer, we have tested the hypothesis that the Src homologyregion 2 domain-containing phosphatase-1 (SHP-1), is a global inhibitor of DC activation and that by blocking SHP-1in DC would induce stronger anti-tumor immunity. Our results demonstrate that inhibition of SHP-1 enhances DCactivation, survival and migration in vitro. Further, using in vivo mouse models, we show that SHP-1 inhibition inDC enhances the generation of CD8+ effector T cells and skews the CD4+ T cell compartment to a Th1 phenotype whileinhibiting the induction of T regulatory cells. These observations suggest that SHP-1 is a pleiotropic inhibitor ofDC function and that its inhibition in DCs enhances the strength of immune responses. Finally, using 2 ectopic mousetumor models (B16 melanoma and TRAMP prostate tumors), we show that SHP-1 inhibition in DC-based vaccinessignificantly inhibits tumor growth. The implication of these data in concert, is that SHP-1 signaling is a feasibleprotein to target in the design and implementation of DC-based vaccines against tumors and potentially against otherinfectious diseases.

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SHP-1 phosphatase, cell-based prostate cancer vaccine, immunotherapy

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INTRODUCTION

An emerging strategy for treatment of late-stage disease is adjuvant stimulation of anti-tumor adaptive immune responses using dendritic cells (DC)[1, 2]. The use of DC to process and present antigen, with or without ectopic expression of various cytokines has shown potential as anti-tumor treatment[3, 4]. Early preclinical and clinical trials suggest that tumor "vaccines" are both feasible and safe[5]. To date clinical trials of anti-tumor "vaccines" have demonstrated only limited efficacy in causing tumor regression despite eliciting measurable systemic T cell responses against prostate cancer[3, 6, 7]. However, these "first-generation vaccines" have given a solid foundation for the use of immunotherapies in the treatment of cancer and provided the impetus to develop the DC vaccine idea further. We proposed modifying DC-based vaccines by genetically altering them to enhance function and to overcome the limitations of the "first-generation vaccines". Our specific approach is to inhibit inhibitors of DC function that normally serve to regulate the initiation of immune responses. We hypothesized that the Src homology region 2 domain-containing phosphatase-1 (SHP-1), is a global inhibitor of DC activation, potentially acting on a number of important signaling pathways, and that by blocking SHP-1 in DC would induce stronger anti-tumor immunity. The experiments described in this report examine the efficacy of knocking down endogenous SHP-1 in mouse bone marrow derived DCs used as vaccines against melanoma and prostate tumors and address the specific mechanisms by which the effects are propagated.

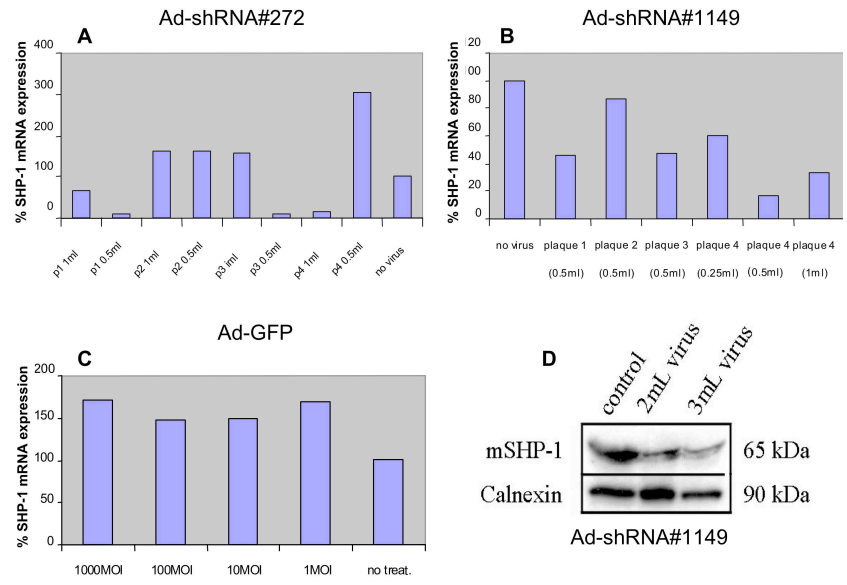

Figure 1. SHP-1 knock-down by shRNA. Adenoviruses expressing either of two shRNAs sequences specific for mouse SHP-1 (sequence 272 or 1149) were plaque purified and the ability to knock-down SHP-1 mRNA was tested in RAW cells by quantitative RT-PCR and are shown as a percent of the no virus treatment A) and B). C) Adenovirus expressing green fluorescent protein was used as a negative control for viral treatment. D) Knock-down of SHP-1 protein by treatment with Ad-shRNA#1149 by western blot with a SHP-1 specific antibody.

BODY OF REPORT

We developed two strategies in order to inhibit SHP-1 function in wild type mouse DCs, small interfering RNA knockdown and over-expression of a phosphatase dead dominant negative mutant.

***SHP-1 specific shRNA* (SOW Task 1a)**

We designed two mouse SHP-1 specific small hairpin RNAs (shRNA) sequences, 272 and 1149, (referred to by their nucleotide position from the start site of the coding sequence in Genbank mRNA Accession # BC012660) and cloned them into the adenoviral vector pAd-BLOCK-iT-DEST RNAi (Invitrogen, Carlsbad, CA) which provides U6 polymerase II promoter-driven expression of the shRNA. Adenovirus carrying the appropriate sequence were produced and expanded in HEK-293 cells (ATCC, Manassas, VA). These viruses were then plaque purified and the ability to knock-down SHP-1 mRNA was tested in RAW 264.7 cells[8], a murine macrophage-like cell line, by quantitative RT-

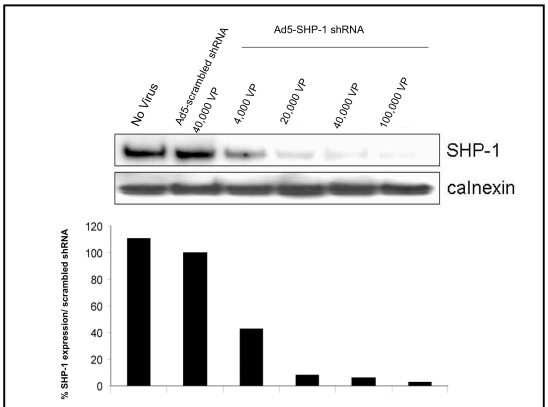

Figure 2. Knock-down of endogenous SHP-1 using high titer Ad5-SHP-1 shRNA. SHP-1 protein expression was measured in D2SC/1 cells following infection with Ad5-SHP-1 at varying doses as shown. Protein levels were analyzed by western blot with a SHP-1 specific antibody. As controls, SHP-1 expression is shown for uninfected cells and those infected with the Ad5-scrambled shRNA. The blot was also probed with a calnexin specific antibody as a loading control. Densitometry analysis of the western is shown below as % SHP-1 expression level relative to cells infected with Ad5-scrambled shRNA.

PCR (Fig. 1A and B). Ad-shRNA#1149 showed the greatest reduction in mRNA (>5 fold reduction) and was selected for subsequent experiments. The ability of this shRNA to knock down SHP-1 protein was examined by western blot (Fig. 1D). A large scale, high titer, preparation of Ad-shRNA#1149 was produced by the Viral Vector Core Laboratory at Baylor College of Medicine and used in subsequent experiments.

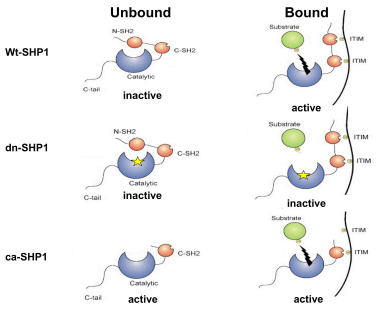

Figure 3. Comparison of SHP-1 mutant and wild type constructs. As a strategy to investigate SHP-1 signaling, we cloned wt-SHP-1 and used it as the basal sequence to engineer dominant negative (dn) and constitutively active (ca) mutants. The dn-SHP-1 mutant is catalytically dead and binds to SHP-1 substrates, blocking endogenous SHP-1 activity. ca-SHP-1 is active with or without binding to immunoreceptor tyrosine-based inhibitory motifs (ITIM). Figure modified from Poole et al. [9]

The high titer adenoviral preparation of SHP-1 specific Ad-shRNA#1149 (referred to from this point on as Ad5-SHP-1-shRNA), produced in the Viral Vector Core Laboratory at Baylor College of Medicine, was tested for its ability to knock-down endogenous SHP-1 protein in D2SC/1 cells, a murine dendritic-like cell line[10]. As a control for adenoviral infection, we used a scrambled shRNA sequence (Ad5-scrambled-shRNA) that showed no significant sequence similarity to any known mouse gene as determined by a BLAST search of the NCBI Genbank nucleotide database. A titration of Ad5-SHP-1 shRNA viral particles was performed and SHP-1 protein expression was determined by western blot (Fig. 2, SOW Task 1c). A 95% decrease in endogenous SHP-1 protein was observed using 40,000 viral particles/cell a viral dose which also resulted in minimal cell death. This dose was chosen for subsequent experiments using mouse bone marrow derived dendritic cells (BMDCs).

Phosphatase dead dominant negative SHP-1 (dn-SHP-1)

We generated the wild type (wt) mouse SHP-1 sequence (wt-SHP-1) by RT-PCR from mouse spleen. Using a splice overlap extension strategy, we mutated the thymine at position 1503 (Genbank Accession # BC012660) to adenosine to create a cysteine to serine point mutant at position 453 (C453S) in the expressed protein that has previously been shown to abolish SHP-1 catalytic phosphatase activity[11]. The C453S mutant has been shown to act as a dominant negative (dn-SHP-1) by competitively binding to SHP-1 substrates and inhibiting endogenous SHP-1 phosphatase activity[11]. In addition to creating the dn-SHP-1 construct to inhibit SHP-1 activity, we also sought to create a control construct in which SHP-1 activity was constitutive. Wild type SHP-1 is inactive in its native conformation due to the N-terminal SH2 domain blocking substrate access to the catalytic site (Fig 3; modified from Poole, 20059). Activation normally requires SH2 domain-dependent binding of SHP-1 to its cognate immunoreceptor tyrosine-based inhibitory motif (ITIM). Thus, we generated a constitutively active (ca) SHP-1 mutant by deleting the N-terminal SH2 domain of SHP-1 that is known to bind and sterically inhibit the catalytic site of SHP-1 when it is not bound to substrate. All three SHP-1 constructs (wt-, dn- and ca-SHP-1) were modified by the addition of a ten amino acid, N-terminal hemagglutinin (HA), coding sequence as an epitope tag to facilitate subsequent detection and differentiation from endogenous SHP-1. These constructs were cloned into the pAdTrack-CMV adenoviral expression vector[12].

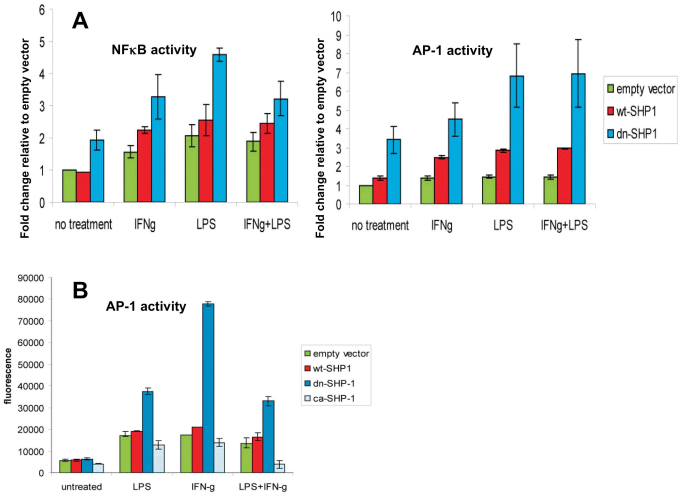

Figure 4. SHP-1 function modulates NFκB and AP-1 signaling. Cells were transfected with a reporter construct for either NFκB or AP-1 and the SHP-1 wt or mutant construct as indicated. Cells were left untreated or incubated with either IFNγ or LPS or both and signaling determined. A and B represent separate experiments and error bars are the standard deviation for 3 replicates per individual treatment within each experiment.

***SHP-1 modulates signaling through multiple receptors in DCs* (SOW Task 1d)**

Function of the mutant and wt SHP-1 constructs was tested in RAW 264.7 cells by transient transfection of SHP-1 vectors along with a reporter construct expressing a secreted alkaline phosphatase driven by either an NFκB or AP-1 dependent promoter. NFκB and AP-1 are major transcription factors stimulated by toll-like receptor (TLR) and cytokine signaling in immune system cells, and represent likely pathways of SHP-1 inhibition on cellular activation. Cells were tranfected with the appropriate construct, reporter and then stimulated with interferon-γ (IFNγ) or bacterial lipopolysaccharide (LPS) as ligands for cytokine and TLR receptors respectively. In all experiments transfection of the dn-SHP-1 construct enhanced both NFκB and AP-1 signaling in response to cytokine or TLR stimulation (Fig 4A), demonstrating that the construct was functional and that SHP-1 normally inhibits these pathways. Transfection with the ca-SHP-1 construct showed the opposite effect to dn-SHP-1 by suppressing AP-1 signaling (Fig 4B), again demonstrating that the construct was functional and that SHP-1 acts on this pathway. All three constructs were sent to the Viral Vector Core Laboratory at Baylor College of Medicine for the production of high titer preparations for use in subsequent experiments.

We also tested a chemical inhibitor of SHP-1 signaling in reporter assays. The inhibitor, sodium stibogluconate (SSG) has been shown to inhibit 99% of SHP-1 enzymatic activity at a concentration of 10μg/ml in cultured cells[13]. D2SC/1 (a mouse dendritic cell line[10]) were transfected with reporter constructs driven by NFκB or AP-1 dependent promoters as above. Transfected cells were grown in media containing 10μg/ml SSG for 2 hrs prior to further supplementation of the media with LPS, IFNγ, CCL21 (a CCR7 chemokine receptor ligand guiding DC migration to the lymph nodes), or IL-10 for 24 hours. SHP-1 inhibition results in increased signaling through TLR4 (LPS), IFNγR, and CCR7 (CCL21) but not through the IL10R (Fig. 5). These data indicate that SHP-1 is active pathways important for DC activation and migration.

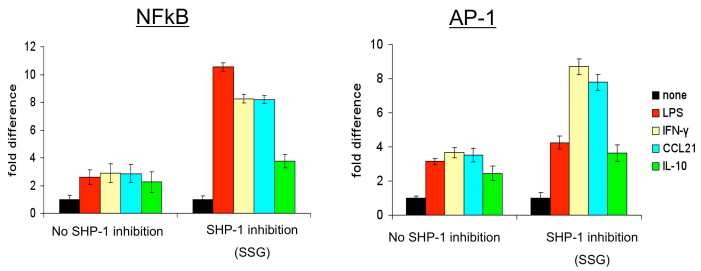

Figure 5. Inhibiting SHP-1 enhances signaling through TLR, IFNγR and CCR7 in DCs. Cells were transfected with a reporter construct for either NFκB or AP-1 and grown in the presence or absence of SSG (a chemical inhibitor of SHP-1 enzymatic activity) as indicated. Cells were left untreated or incubated with LPS, IFNγ, CCL21 or IL-10. Signaling was determined by fluorescent detection of the secreted alkaline phosphatase expressed by the reporter constructs and expressed as a fold change over no SHP-1 inhibition. Error bars are the standard deviation for 3 replicates per individual treatment within each experiment.

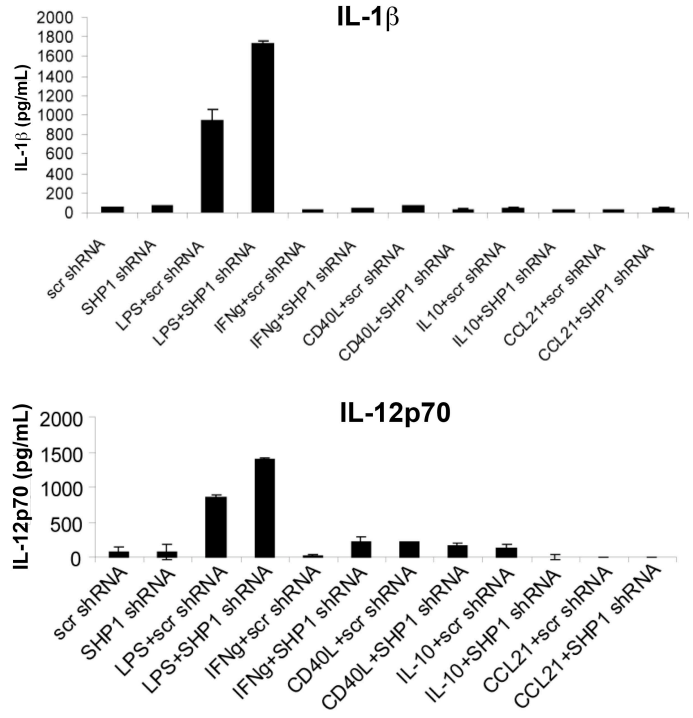

Figure 6. Inhibition of SHP-1 in DCs enhances IL-1β and IL-12 expression. BMDCs were transduced with Ad5-SHP-1-shRNA or Ad5-scrambled-shRNA and stimulated with one of five stimuli, LPS, IFNγ, CD40L, IL-10, or CCL21, or left untreated. Following 24 hours of stimulation the supernatant was collected and analyzed using a luminex bead array. Expression of IL-1β and IL-12p70 (the active heterodimer of IL-12) are shown in pg/ml supernatant. Error bars represent the standard deviation of 2 replicate wells. These data are representative of 3 separate experiments each showing similar results.

In a recent publication, An and colleagues[14] showed significant qualitative differences in immune signaling when SHP-1 was inhibited by RNAi knockdown compared with inhibition by dominant negative SHP-1 expression. These experiments may be critical in determining which method of SHP-1 inhibition we will ultimately utilize as

our tumor vaccine. Thus, through out this report we utilize SHP-1 inhibition thorough several different methods both in *in vitro* experiments and in *in vivo* vaccine experiments from SOW Task 2.

SHP-1 affects IL-12 and IL-1β expression

To determine the affect of SHP-1 inhibition on DC cytokine production, BMDCs were transduced with Ad5-SHP-1-shRNA or Ad5-scrambled-shRNA (as a negative control for adenoviral transduction) and stimulated with one of five different stimuli, LPS, IFNγ, CD40L, IL-10, or CCL21, representing major activation signaling pathways. DCs were incubated for 24 hours with the individual stimuli and the media was collected for analysis by a luminex-based bead array assay for the following cytokines: IL-1β, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-15, TNFα, and IFNγ. LPS signaling through TLR4 proved the strongest stimulus and elicited a dramatic increase in the expression of IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, and TNFα. No stimulus elicited increases in IL-4, IL-15 or IFNγ. SHP-1 knockdown enhanced the LPS induced expression of IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, and TNFα. SHP-1 inhibition al enhanced the IFNγ stimulated expression IL-6, IL-12p40 and IL-12p70. Figure 6 shows the results of the screening for IL-1β and IL-12p70 (the active heterodimer of IL-12) from all stimulations as an example. Additionally we have repeated these exact experiments using either SSG or the Ad5-DN-SHP-1 construct as the method of SHP-1 inhibition with similar results to that seen with the Ad5-SHP-1-shRNA. IL-1β and IL-12 are major inflammatory cytokines that are necessary for the initiation of $CD8^+$ T cell responses. Taken together, these data suggest that SHP-1 negatively regulates activation pathways in DCs that are necessary for the initiation of anti-pathogen and potentially anti-tumor T cell responses. It further suggests that by inhibiting SHP-1 in DCs we will enhance anti-tumor vaccinations.

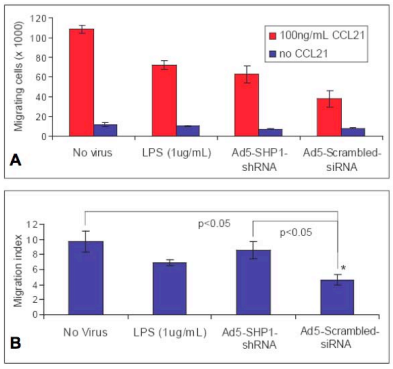

Figure 7. SHP-1 inhibits CCR7-dependant migration *in vitro*. $5x10^5$ BMDCs were transduced with Ad5-SHP-1-shRNA or Ad5-scrambled-shRNA or left untreated, and placed in the upper chamber of a transwell containing a membrane with 5 µm pores. Transduced cells or those treated with LPS as a control, were placed over a bottom chamber filled with either media alone or media containing 100ng/mL CCL21 and incubated at 37˚C for 3 hours. A) the number of DCs migrating to the bottom well. B) the migration index for each treatment group (number migrating with CCL21/number migrating with media alone). Statistical differences were between groups were determined by t test. Error bars represent the standard error of the mean of 3 replicate wells for each treatment. These data are from one of 3 separate experiments each showing similar results.

***SHP-1 modulates DC migration both* in vitro *and* in vivo (SOW Task 1e)**

For all subsequent experiments described in this report, we prepared primary bone marrow-derived dendritic cells (BMDCs) from wild type mice in the following manner: Bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice and cultured for 6 days in RPMI media supplemented with 10% FBS, 10ng/mL IL-4 and 10ng/mL GMCSF along with antibiotics. On day 6, dendritic cells (DCs) were either purified by magnetic bead assisted cell sorting (MACS; Miltenyi Biotec, Auburn, CA) prior to adenoviral transduction, or used as unpurified bulk DCs that were then transduced with adenovirus.

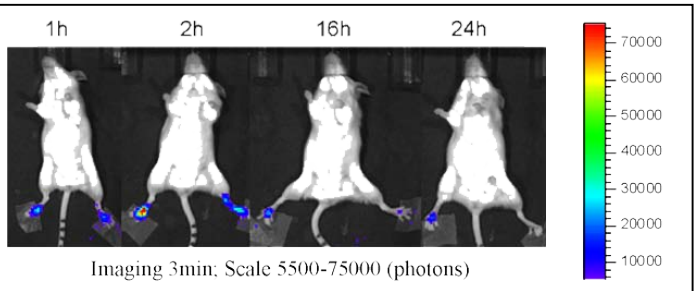

Figure 8. Knock down of SHP-1 enhances the migration of DCs out of the footpad. $2 x10^6$ DCs transduced with either Ad5-SHP-1-shRNA (right side footpad) or Ad5-scrambled-shRNA (left side footpad) were injected into the footpads of wt C57BL/6 mice. At timed intervals mice were injected i.p. with 100 µl of 10 mg/mL luciferin and imaged *in vivo* using IVIS® 10 minutes later. Shown is one representative image from 5 mice.

For DCs to initiate an immune response they must capture antigen in the periphery and then migrate to the lymph nodes where they stimulate antigen specific T cells. To determine if SHP-1 signaling could affect the ability of DCs to migrate to draining lymph nodes, we performed trafficking experiments both *in vitro* and *in vivo*. For the *in vitro* DC migration assays, unpurified bulk BMDCs were transduced with either Ad5-SHP1-shRNA or Ad5-scrambled-shRNA for 48h or left untreated. Half of the untreated cells were treated with 1µg/mL LPS for 24h. Cells were collected and washed in serum-free media (SFM) and resuspended

at 5 x 10^6 cells/mL. 500µL of SFM containing 100ng/mL CCL21, a CCR7 ligand and one of the chemokines responsible for DC trafficking to lymph nodes *in vivo*, was used as the trafficking media and was added to the bottom chamber of a 24-well transwell plate (5µm pores). Wells loaded with 500µL of SFM without CCL21 were used as control for basal migration. 100µL of the BMDC suspension was loaded in upper chamber of each transwell and was placed over the chambers containing the appropriate trafficking media. Transwell plates were incubated at 37˚C for 3h. BMDCs migrating into the lower chamber were counted using a hematocytometer.

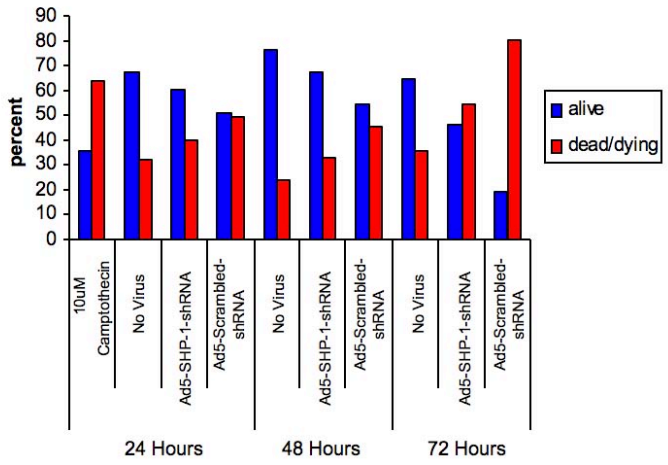

Figure 9. SHP-1 knock down enhances DC survival. Purified DCs were transduced with either Ad5-SHP1-shRNA or Ad5-scrambled-shRNA or left untreated and the virus was washed away following a 2 hour exposure. Cell survival was determined at 24h, 48h and 72h after infection by annexin V and propidium iodide (PI) staining and analyzed by flow cytometry. Camptothecin treatment for 24 hours was used as a positive control for apoptosis. Cells were considered alive if they were negative for both annexin V and PI. All cells that were positive for either annexin V or PI or both were considered dead or dying.

In all treatments exposure to CCL21 in the lower chamber markedly enhanced the rate of migration of DCs (Fig. 7A). Exposure of mouse BMDCs to LPS is known to cause a decrease in the rate of migration to CCR7 ligands, an effect that differs from that seen in human DCs[15]. Similarly, exposure to adenovirus has also been shown to reduce the rate of migration in murine DCs15. When BMDCs were transduced with Ad5-SHP-1-shRNA, their migration rate to CCL21 was not significantly different from that of untreated cells when compared to the background rate of migration in the absence of CCL21 (the migration index; Fig. 7B). This was in contrast to DCs transduced with the Ad5-scrambled-shRNA virus, which showed a significant reduction in migration index compared to both untreated cells and those treated with Ad5-SHP-1-shRNA (Fig. 7B).

To determine if SHP-1 inhibition enhanced DC migration *in vivo*, BMDCs were prepared as described above but were transduced with an adenovirus expressing a clickbeetle red-shifted luciferase in addition to either Ad5-SHP-1-shRNA or Ad5-scrambled-shRNA. 2 x10^6 transduced cells were injected into the contralateral footpads of tyrosinase-deficient albino C57BL/6 mice (B6(Cg)-$Tyr^{c\text{-}2J}$/J, Jackson Laboratory). At the specified intervals mice were injected i.p. with 100 µl of 10 mg/mL luciferin and imaged *in vivo* using IVIS® (Caliper Life Sciences, Hopkinton, MA). DCs treated with Ad5-SHP-1-shRNA could be seen to migrate out of the footpad by 2 hours post-injection and undetectable in the footpad by 24 hours post injection (Fig 8 right side footpad). In contrast, DCs treated with the Ad5-scrambled-control-shRNA were still evident in the footpad at only marginally reduced levels even at 24 hours post-injection (Fig 8 left side footpad). Taken together, these data indicate that SHP-1 modulates chemotaxis in mouse BMDCs and that inhibition of SHP-1 signaling enhances DC migration.

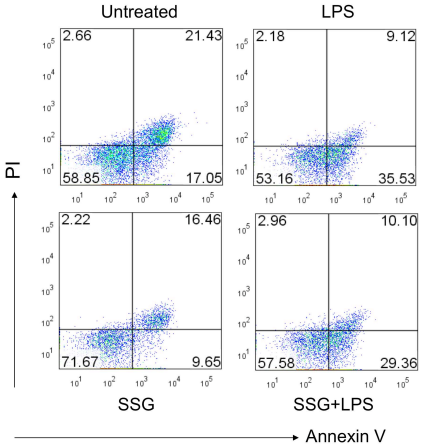

Figure 10. SHP-1 knock down enhances DC survival. Purified DCs were incubated in the presence of absence of LPS and/or SSG for 48 hours. Cell survival was determined by annexin V and propidium iodide (PI) staining and analyzed by flow cytometry. Cells were considered alive if they were negative for both annexin V and PI. All cells that were positive for either annexin V or PI or both were considered dead or dying.

SHP-1 modulates DC survival

BMDCs were prepared as described above and purified by CD11c (a marker for DC) MACS. Purified DCs were transduced with either Ad5-SHP1-shRNA or Ad5-scrambled-shRNA or left untreated and the virus was washed away following a 2 hour exposure. Cell survival was determined at 24h, 48h and 72h after infection by annexin V and propidium iodide (PI) staining and

analyzed by flow cytometry. Annexin V binds to phosphatidylserine and is an early marker of apoptosis. PI is a DNA intercalating dye that can only enter cells when their membrane is integrity is disrupted and is a marker of cells late in the apoptotic process. Viral infection of DCs causes cells to undergo apoptosis where approximately 50% were dead or dying within the first 24 hours and 85% were dead or dying by 72 hours post-infection (Fig. 9 Ad5-scrambled-shRNA). In contrast, cells treatment of with Ad5-SHP-1-shRNA showed greater viability with only 35% dead or dying within the first 24 hours and 55% dead or dying by 72 hours post-infection (Fig. 9 Ad5-SHP-1-shRNA). For cells not treated with virus 35% were dead or dying after 72 hours.

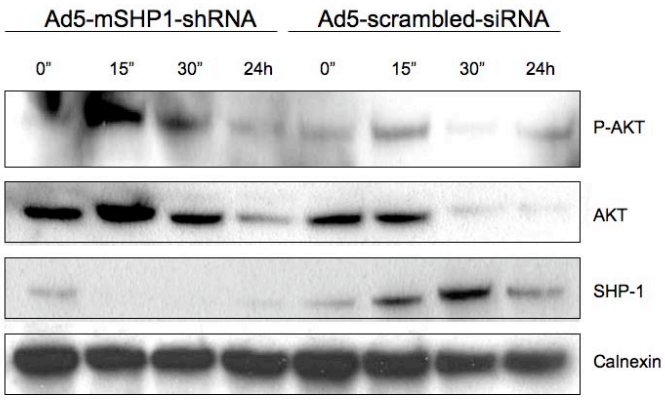

Figure 11. SHP-1 signaling inhibits Akt phosphorylation. CD11c positive BMDCs were transduced with either Ad5-SHP-1-shRNA or Ad5-scrambled-shRNA or left untreated. Cells were treated with 1µg/mL LPS for the times indicated and the resulting cell lysates analyzed by western blot for phosphorylated Akt (p-Akt). Blots were stripped and reprobed as indicated for total Akt, SHP-1, and the ubiquitous ER chaperone, calnexin, as a loading control.

We repeated this experiment using SSG as the method for inhibiting SHP-1 in DCs. DCs were incubated in the presence of absence of LPS and/or SSG for 48 hours and cell death was measured as positive annexin V and PI staining as above. LPS induces apoptosis in DCs under these assay conditions (Fig. 10 upper right panel). Inhibition of SHP-1 with SSG increased the annexin V negative/PI negative population from 59% to 72% and additionally inhibited the effect of LPS treatement on DCs. Taken together these data and those in Fig. 9, indicate that SHP-1 signaling can promote apoptosis in DCs and that inhibiting SHP-1 leads to enhanced survival.

DC survival has been linked with activation of Akt/protein kinase B (PKB) family proteins, major effectors of phosphatidylinositol 3-kinase (PI3K) family members[16, 17]. We examined LPS stimulated Akt signaling BMDCs to determine if SHP-1 mediated survival might be working through this mechanism. MACS sorted for CD11c positive BMDCs were infected with either Ad5-SHP-1-shRNA or Ad5-scrambled-shRNA or left untreated. 10^7 cells per group were treated with 1µg/mL LPS for the times indicated or left untreated. Lysates were analyzed by western blot for phosphorylated Akt. SHP-1 knock down enhanced LPS mediated Akt phosphorylation and also enhanced the steady state expression of total Akt protein (Fig. 11). This observation suggests a mechanism for the increased survival seen when SHP-1 signaling was inhibited (Fig. 9 and 10). LPS stimulation also appears to enhance the total level of SHP-1 protein in DCs as shown by the SHP-1 blot for cells treated with Ad5-scrambled-shRNA. Taken together these data indicate that SHP-1 signaling can promote apoptosis in DCs through the inhibition of Akt phosphorylation and that inhibiting SHP-1 leads to enhanced DC survival.

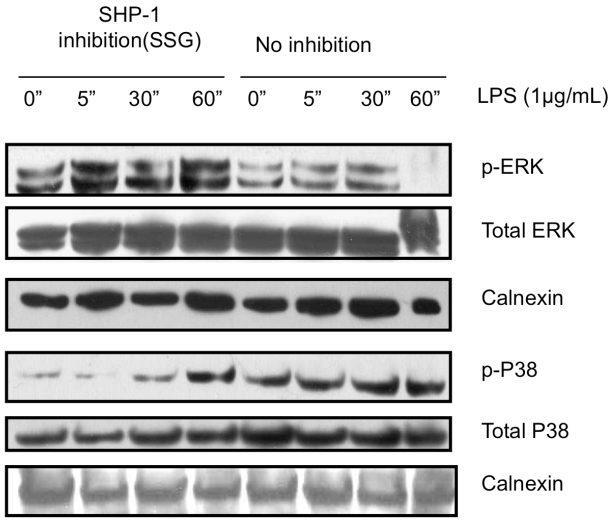

Figure 12. SHP-1 signaling inhibits ERK and p38 MAP kinase phosphorylation . CD11c positive BMDCs were transduced with either Ad5-SHP-1-shRNA or Ad5-scrambled-shRNA or left untreated. Cells were treated with 1µg/mL LPS for the times indicated and the resulting cell lysates analyzed by western blot for phosphorylated ERK 1/2 (p-ERK) or p38 (p-P38)MAP kinases. Blots were stripped and reprobed as indicated for total ERK, p38, and the ubiquitous ER chaperone, calnexin, as a loading control.

Survival and activation signaling in many cell types have been shown to be transduced through the mitogen-activated protein kinases (MAP kinases) p38 and ERK. We investigated if SHP-1 affected p38 or ERK signaling pathways. BMDCs were treated with SSG or left untreated. 10^7 cells per group were treated with 1µg/mL LPS for the times indicated or left untreated. Lysates were analyzed by western blot for phosphorylated ERK or p38. SHP-1 inhibition enhanced LPS mediated ERK phosphorylation had no effect on the steady state expression of total ERK protein (Fig. 12). Conversely, SHP-1 inhibition decreased total p38 expression and markedly enhanced the phosphorylation of p38 (Fig. 12). These data indicate that signals transduced through these MAP kinases are modulated to some extent by SHP-1 and that inhibiting SHP-1 activity we can enhance these activation signals. Taken together the increases in Akt and MAPK signaling suggest a mechanism for the increased survival and potentially increased DC activation that may ultimately lead to increases in T cell activation.

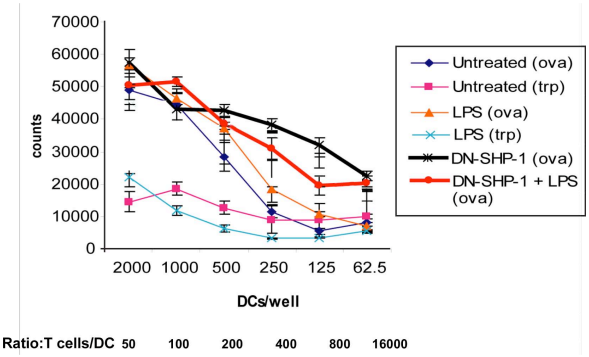

Figure 13. SHP-1 knock down enhances CD8$^+$ effectors and CD4$^+$ Th1 while inhibiting FOXP3$^+$ Treg induction. BMDCs were loaded with either the ovalbumin peptide, SIINFEKL (the active peptide recognized by OT1 transgenic T cells), or an irrelevant peptide from B16 melanoma cells Trp-2 (SVYDFFVWL). Following peptide loading DCs were left untreated or transduced with an adenoviral vector expressing the dominant negative SHP-1 construct (Ad5-DN-SHP-1). Subsequently DCs were treated with LPS or left untreated and plated with splenocytes from OT1 transgenic mice. Cells were incubated overnight before the addition of ^{3}H-thymadine for a further 24 hours to detect proliferation. Proliferation of T cells is shown as radioactive counts (^{3}H-thymadine uptake). viral treatment. Error bar represent the standard deviation of 3 replicate wells. This is representative of 2 independent experiments.

SHP-1 inhibition in DCs enhances T cell stimulation in vitro (SOW Task 1g)

To determine if SHP-1 inhibition in DCs resulted in an increased ability to stimulate T cell activation, BMDCs were loaded with either the ovalbumin peptide, SIINFEKL (the active peptide recognized by OT1 transgenic T cells), or an irrelevant peptide from B16 melanoma cells Trp-2 (SVYDFFVWL). Following peptide loading DCs were left untreated or transduced with an adenoviral vector expressing the dominant negative SHP-1 construct (Ad5-DN-SHP-1). Subsequently DCs were treated with LPS or left untreated and then plated with splenocytes from OT1 transgenic mice. Cells were allowed to interact overnight before being pulsed with ^{3}H-thymadine for a further 24 hours. OT1 transgenic mice express a transgenic T cell receptor that recognizes chicken ovalbumin peptide, SIINFEKL (OVA), in the context of the MHC class I molecule K^b. OT1 T cells were incubated with decreasing numbers of DCs per well from 2000DC/well to 62.5 DCs/well (effective T cell ratios from 50 T cells/DC - 16000 T cells/DC respectively as shown in Fig. 13).

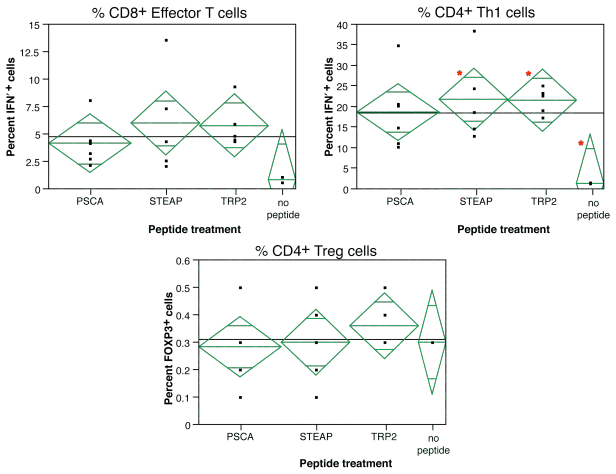

Figure 14. DC vaccination enhances Th1 skewing of T cells. BMDCs vaccines were loaded with one of 3 different peptide tumor antigens: 1) Trp-2 (SVYDFFVWL); 2) STEAP$_{327\text{-}335}$ (VSKINRTEM; or 3) PSCA$_{29\text{-}37}$ (AQMNNRDCL). Following peptide loading DCs were left untreated or transduced with one of 3 different adenoviral vectors: 1) Ad5-SHP-1-shRNA; 2) Ad5-scrambled-shRNA; or 3) Ad5-CMV-empty, an adenovirus carrying a CMV promoter expression vector but no insert. Vaccines were injected i.p. into wt C57BL/6 mice and T cell skewing analyzed 7 days later by flow cytometry from total splenocytes. Cells were stained with anti-CD3, anti-CD8 and anti IFNγ for effector and Th1 cells or anti-CD4 and anti-FOXP3 for Tregs. For the peptide specific analysis viral treatments were pooled for each peptide exposure. Data represent the averages 6 mice per peptide treatment and 2 mice in the no treatment control group. The green diamonds represent the mean and 95% confidence interval for each group from the ANOVA. The black line is the mean of means for the experiment and red * indicate significant differences ($p<0.05$ by Tukey-Kramer HSD multiple comparisons test)

Dendritic cells loaded with the Trp-2 peptide did not stimulate OT1 proliferation. High numbers of DCs loaded with the OVA stimulated OT1 proliferation at similar levels irrespective of DC treatment (LPS or DN-SHP-1). This indicates that the presence of a sufficient amount of antigen can over-ride interactive activation constraints between DCs and T cells. However, when 250 or fewer DCs were added per well, SHP-1 inhibited DCs stimulated significantly higher OT1 proliferation that either untreated DCs or DCs treated with LPS alone. We have repeated these experiments using SSG treated DCs instead of Ad5-DN-SHP-1 DCs with similar results (data not shown). These data demonstrate that SHP-1 inhibition in DCs increases their ability to activate T cell responses.

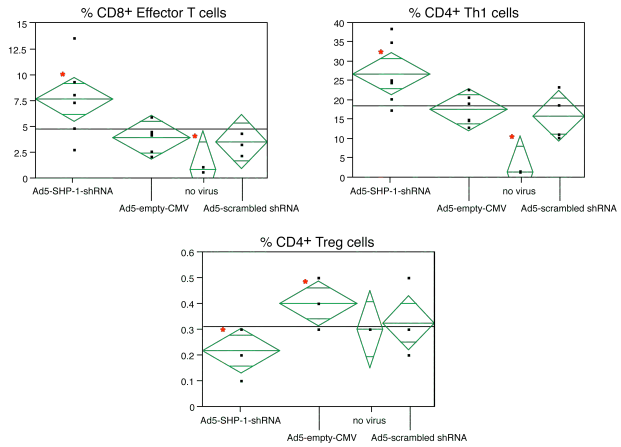

Figure 15. SHP-1 knock down enhances CD8$^+$ effectors and CD4$^+$ Th1 while inhibiting FOXP3$^+$ Treg induction. BMDCs vaccines were loaded with one of 3 different peptide tumor antigens: 1) Trp-2 (SVYDFFVWL); 2) $STEAP_{327-335}$ (VSKINRTEM; or 3) $PSCA_{29-37}$ (AQMNNRDCL). Following peptide loading DCs were left untreated or transduced with one of 3 different adenoviral vectors: 1) Ad5-SHP-1-shRNA; 2) Ad5-scrambled-shRNA; or 3) Ad5-CMV-empty, an adenovirus carrying a CMV promoter expression vector but no insert. Vaccines were injected i.p. into wt C57BL/6 mice and T cell skewing analyzed 7 days later by flow cytometry from total splenocytes. Cells were stained with anti-CD3, anti-CD8 and anti IFNγ for effector and Th1 cells or anti-CD4 and anti-FOXP3 for Tregs. For the SHP-1 specific analysis viral treatments were pooled for each viral treatment. Data represent the averages 6 mice per peptide treatment and 2 mice in the no treatment control group. The green diamonds represent the mean and 95% confidence interval for each group from the ANOVA. The black line is the mean of means for the experiment and red * indicate significant differences ($p<0.05$ by Tukey-Kramer HSD multiple comparisons test).

***SHP-1 knock down enhances CD8$^+$ effectors and CD4$^+$ Th1 while inhibiting FOXP3$^+$ Treg induction* in vivo (SOW Task 1g)**

To determine the effect of SHP-1 inhibition on the initiation of T cell responses, BMDCs were cultured and CD11c$^+$ MACS purified as described above. DCs were loaded with one of 3 different peptide tumor antigens: 1) tyrosinase-related protein 2 ($TRP\text{-}2_{181-188}$; SVYDFFVWL) that binds to H-2K^b and is specific for the B16 murine melanoma tumor line[18]; 2) six transmembrane antigen of the prostate ($STEAP_{327-335}$; VSKINRTEM) that binds to H-2D^b and is specific for transgenic adenocarcinoma of the mouse prostate (TRAMP) tumors (we defined this epitope, see tumor experiments below); or 3) prostate stem cell antigen ($PSCA_{29-37}$; AQMNNRDCL) that binds to H-2D^b and is specific for TRAMP tumors (we also defined this epitope, see tumor experiments below). Following peptide loading DCs were left untreated or transduced with one of 3 different adenoviral vectors: 1) Ad5-SHP-1-shRNA; 2) Ad5-scrambled-shRNA; or 3) Ad5-CMV-empty, an adenovirus carrying a CMV promoter expression vector but no insert. Ad5-CMV-empty was used as an additional negative control to the Ad5-scrambled-shRNA virus, to demonstrate that Ad5-scrambled-shRNA did not have any specific RNAi activity that might facilitate DC inhibition. Using all combinations of peptide and virus treatment yielded 9 experimental vaccines and one no vaccine control. Vaccines ($2x10^6$ DCs) were injected i.p. into wt C57BL/6 mice. Seven days following vaccination mice were sacrificed and total splenocytes were analyzed by multi-color flow cytometry for the expression of several T cell subsets. CD3$^+$CD8$^+$IFNγ$^+$ cells were characterized as CTL effectors, CD3$^+$CD4$^+$IFNγ$^+$ cells were characterized as Th1 helper T cells, and CD4$^+$FOXP3$^+$ cells were characterized as Treg cells. Flow cytometry data were analyzed by one-way analysis of variance (ANOVA) and Tukey-Kramer HSD multiple comparisons test for percentage of cells falling within each population following treatment (Fig. 14). Cells were stained with anti-CD3, anti-CD8 and anti IFNγ to differentiate CD8$^+$ effectors and CD4$^+$ Th1 T cell skewing or anti-CD4 and anti-FOXP3 to determine Tregs. To determine if there was a peptide specific effects between the 3 different tumor antigens, viral treatments were pooled for each peptide exposure. Data represent the averages 6 mice per peptide treatment group and 2 mice in the no treatment control group. No significant differences were seen between peptides in the induction of Tregs or CD8$^+$ effector cells. Th1 skewing was significant between STEAP and Trp-2 peptides and the no treatment control (ANOVA: df=3, F=3.67, $p<0.05$ Tukey-Kramer HSD $q^*=2.91$, $p<0.05$) suggesting that DC vaccination with any peptide combination induces a Th1 response.

To determine if there was a SHP-1 specific effect between the viral treatments, peptide treatments were pooled for each viral exposure. Data represent the averages 6 mice per viral treatment group and 2 mice in the no treatment control group (Fig. 15). SHP-1 specific knock down with Ad5-SHP-1-shRNA induced a significantly higher proportion of $CD8^+$ effector cells control (ANOVA: df=3, F=5.06, p<0.02 Tukey-Kramer HSD q*=2.91, p<0.05) and $CD4^+$ Th1 skewed T cells control (ANOVA: df=3, F=9.01, p<0.002 Tukey-Kramer HSD q*=2.91, p<0.05) compared to the untreated (no vaccine) control. Treatment with the Ad5-scrambled-shRNA and the Ad5-CMV-empty controls showed a trend towards increased $CD8^+$ effector cells and $CD4^+$ Th1 T cells but these increases were not significantly different from either the no treatment control or the Ad5-SHP-1-shRNA treated vaccines. Examination of the effect of SHP-1 knock down on the induction of Tregs showed that inhibition of SHP-1 significantly decreased the percentage of Tregs compared to the empty viral control control (ANOVA: df=3, F=3.50, p<0.05 Tukey-Kramer HSD q*=2.91, p<0.05). No significant differences were seen between the control virus treated groups or between the control treated and the untreated controls. Taken together these data indicate that inhibiting SHP-1 in DC vaccines significantly increases the induction of CTL responses and Th1 skewing suggesting the likelihood of an enhanced anti-tumor immune response. Supporting this, is the fact that in addition to effector $CD8^+$ CTL increases, SHP-1 correspondingly diminishes the suppressive Treg response suggesting an even greater anti-tumor effect may be achieved.

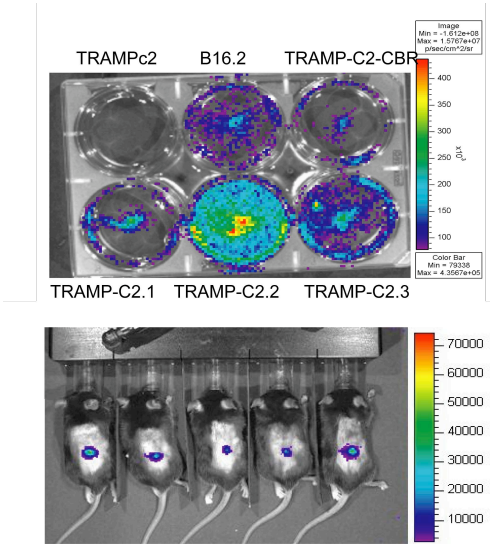

Figure 16. Luciferase expressing glow tumors. Clones of luciferase expressing B16 and TRAMP C-2 tumor lines growing in vitro (upper panel). TRAMP C-2 glow tumors were injected s.c. into C57BL/6 mice and are shown 3 days post-injection of $7x10^6$ cells using IVIS® imaging (Caliper Life Sciences, Hopkinton, MA)

Creating tumor cell lines stably expressing red-shifted luciferase for* in vivo *imaging of ectopic and metastatic tumors in live animals (SOW 2a)

We have created tumor lines that stably express a red-shifted luciferase in order to allow us to monitor the size and location of model tumors in living animals using IVIS™ optical bioluminescence imaging. These various tumor lines were transfected with a luciferase expression vector, cloned by limiting dilution and selected for the brightest expression when exposed to the substrate luciferin (Fig. 16). As a proof of principle for using these tumor lines, we tested the luciferase-transfected B16 and TRAMP C-2 tumor lines for growth in wt C57BL/6 mice. These results showed that luciferase-transfected tumors grew substantially slower that the untranfected tumors *in vivo*. In addition, the transfected tumor lines were for the most part resolved by the animals in the absence of any vaccination. Thus we have not proceeded further with these glow tumors for our experiments and have gone back to using the original tumor lines and taking physical measurements using calipers for all tumor experiments described below.

SHP-1 knock down enhances DC vaccine efficacy against B16 melanoma and TRAMP C-2 prostate tumors (SOW 2b)

Since we had shown that SHP-1 inhibition enhanced DC activation signaling, migration, survival, and $CD8^+$ effector function, we wanted to determine if inhibiting SHP-1 in DCs would enhance their function as anti-tumor vaccines. To test this in a prostate cancer model in vivo we used TRAMP C2 cells injected

Peptide Epitope	AA Sequence	Bimas*	SYFPEITHI
OVA258-265	SIINFEKL	17.4 (Kb)	25 (Kb)
STEAP186-192	RSYRYKLL	132 (Kb)	29 (Kb)
STEAP84-91	LTFLYTLL	48 (Kb)	22 (Kb)
STEAP327-335	VSKINRTEM	718.829 (Db)	26 (Db)
STEAP262-270	LLLGTVHAL	4.311 (Db)	12 (Db)
PSCA29-37	AQMNNRDCL	10838.473 (Db)	25 (Db)

Table 1. OVA Kb binding peptide SIINFEKL was used as control for a known good binder. 4 peptides were chosen from the STEAP-1 protein that showed a high ranking for predicted binding affinity or showed sequence homology to the known human HLA-A*0201 binding epitopes from human STEAP-1 (suggesting the peptide is likely to be processed *in vivo* and one PSCA peptide was chosen because of its strong predicted binding affinity.

subcutaneously on the dorsal flank of C57BL/6 mice. Unfortunately, there have been no good tumor antigens previously defined for the TRAMP model. Recent studies have shown, however, that the six transmembrane epithelial antigen of the prostate (STEAP) is a good candidate for immunotherapies in human prostate cancer[19]. In addition, a recent study showed that the mouse homolog of STEAP (which is 80% identical to the human protein) and the mouse homolog of prostate stem cell antigen (PSCA) were expressed at high levels in TRAMP C2 cells[20]. To determine peptide epitopes from these proteins that were potentially immunoreactive, used two online epitope prediction algorithms Bimas (www-bimas.cit.nih.gov) and SYFPEITHI (www.syfpeithi.com) to scan their amino acid sequences. Predictions of peptides binding to the MHC class I molecules H-2K^b and H-2D^b from the C57BL/6 background, yielded a number of candidate epitopes. Of these candidates, we chose epitopes that were either the strongest predicted binders or that had sequences similar to published human epitopes and therefore suggested that they were likely to be processed *in vivo*. The five epitopes chosen for testing are shown in Table 1 and include OVA peptide, as a comparison for strong H-2K^b binding. We tested the binding affinity of the five peptide we had predicted, in a surface stabilization assay, using the TAP1 deficient cell line RMA-S[21]. RMA-S cells were pulsed with peptide, at the indicated concentrations, and incubated overnight. Cells were stained with antibodies for H-2K^b (Y-3; ATCC-HB176) or H-2D^b (28-14-8S; ATCC-HB27) followed by a fluorescent-labeled secondary antibody and MHC class I surface expression was analyzed by flow cytometry (Fig. 17). All peptides bound to their expected class I molecules with the exception of STEAP$_{262-270}$ that showed no detectable binding at any peptide concentration. STEAP$_{84-91}$ and STEAP$_{186-192}$ bound with an affinity near that of OVA$_{258-265}$, a well characterized strong binding H-2K^b epitope from chicken ovalbumin. Although PSCA$_{29-37}$ and STEAP$_{327-335}$ did not appear to bind as well as some of the other peptides they are predicted to bind to H-2D^b not H-2K^b. Because we did not have a positive control strong binding peptide for H-2D^b in these experiments, we cannot rule out the possibility that the "lower" binding affinity of these peptides may be due to a lower relative expression of H-2D^b on RMA-S cells compared to H-2K^b, or differences in the binding affinities of the different antibodies use to detect each molecule. Taken together, these data suggest that at least 4 of the 5 predicted peptide epitopes for TRAMP C2 tumors could act as immunoreactive antigens when administered *in vivo* as part of an anti-tumor vaccine.

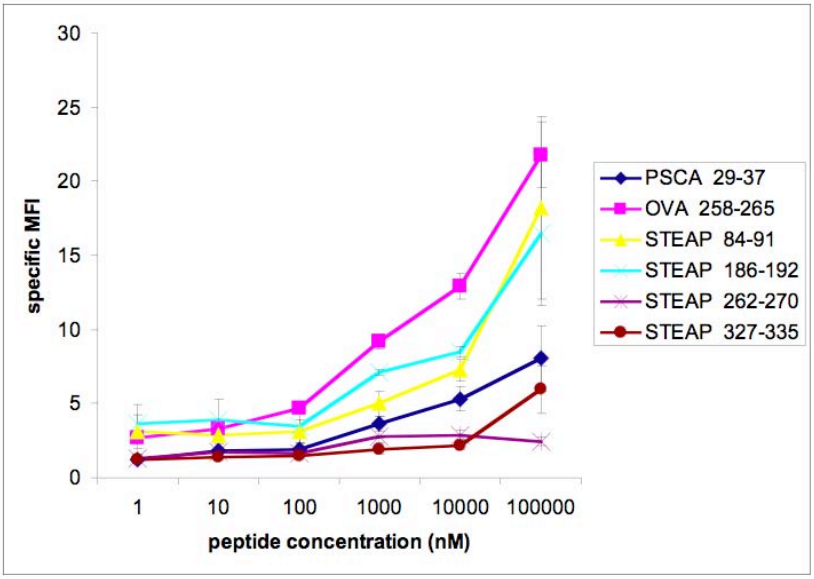

Figure 17. Relative binding affinities of STEAP and PSCA peptides predicted to bind H-2K^b or H-2D^b. 10^6 RMA-S cells were incubated overnight at 37˚ C with each of the five predicted at the concentrations indicated. Cells were stained with antibodies for H-2K^b (Y-3; ATCC-HB176) or H-2D^b (28-14-8S; ATCC-HB27) followed by a goat anti-mouse-FITC second step and MHC class I surface expression was analyzed by flow cytometry. Specific MFI is the mean fluorescent intensity of the sample- mean fluorescent intensity of goat anti-mouse-FITC second step alone. Error bars represent the standard deviation of triplicate measurements. This is representitive of 3 separate experiments.

To determine the efficacy of the newly predicted TRAMP C2 peptide antigens, BMDCs were prepared as described above, transduced with either Ad5-SHP-1-shRNA or the control Ad5-scrambled-shRNA at 40,000 viral particles/ cell, and pulsed with one of the six peptides listed in Table 1 (OVA, an irrelavent epitope to TRAMP 2 tumors, served as a negative control peptide) or a lysate of TRAMP C2 cells as a positive control antigen. 6-8 week C57BL/6 mice, bearing TRAMP C2 tumors ($7x10^6$ cells injected s.c. on the dorsal flank three days prior to vaccination), were given a single i.p. vaccination with $2x10^6$ treated DCs or left untreated. Tumors were measured (length and width using calipers) every 2-4 days until termination of the experiment and tumor volume was estimated using the formula: Tumor volume (mm^3) = x^2y x 0.5236, where x is the smaller tumor dimension, and y is the larger tumor dimension.

In mice not receiving any vaccination ectopic TRAMP C2 tumors grow exponentially and reach maximum allowable size (10% of body weight, 2000-3000 mm^3 depending on the age of the mice) in 75-85 days (determined empirically from pilot experiments, data not shown). The growth rate of ectopic TRAMP C2 tumors in mice vaccinated with DCs loaded with the irrelevant control peptide, OVA, was equivalent to mice receiving no vaccine treatment (Fig. 18A). These data indicate that DCs alone in the absence of a cognate tumor antigen cannot stimulate an anti-tumor response. Mice vaccinated with DCs loaded with one of the STEAP or PSCA peptides or the TRAMP C2 lysate, all showed a similar trend of decreased tumor growth rate compared to the untreated mice (Fig. 18B-G). On closer inspection of the differences between the mean tumor volumes (5 mice/treatment), two peptides generated strong inhibition of tumor growth, $PSCA_{29-37}$ and $STEAP_{327-335}$ (Fig. 18E and F). To determine if there was a significant difference between the various peptides used in these DC vaccinations, we pooled mice across viral treatments (Ad5-SHP-1 shRNA or Ad5-scrambled-shRNA) employing the same peptide. We performed an ANOVA for differences in mean tumor volume at day 48 post-vaccination (the time at which the majority of the vaccinated tumor growth curves change slope) and Tukey-Kramer HSD multiple comparisons test. A significant difference was seen between peptide treatments (ANOVA: df=7, F=3.80,

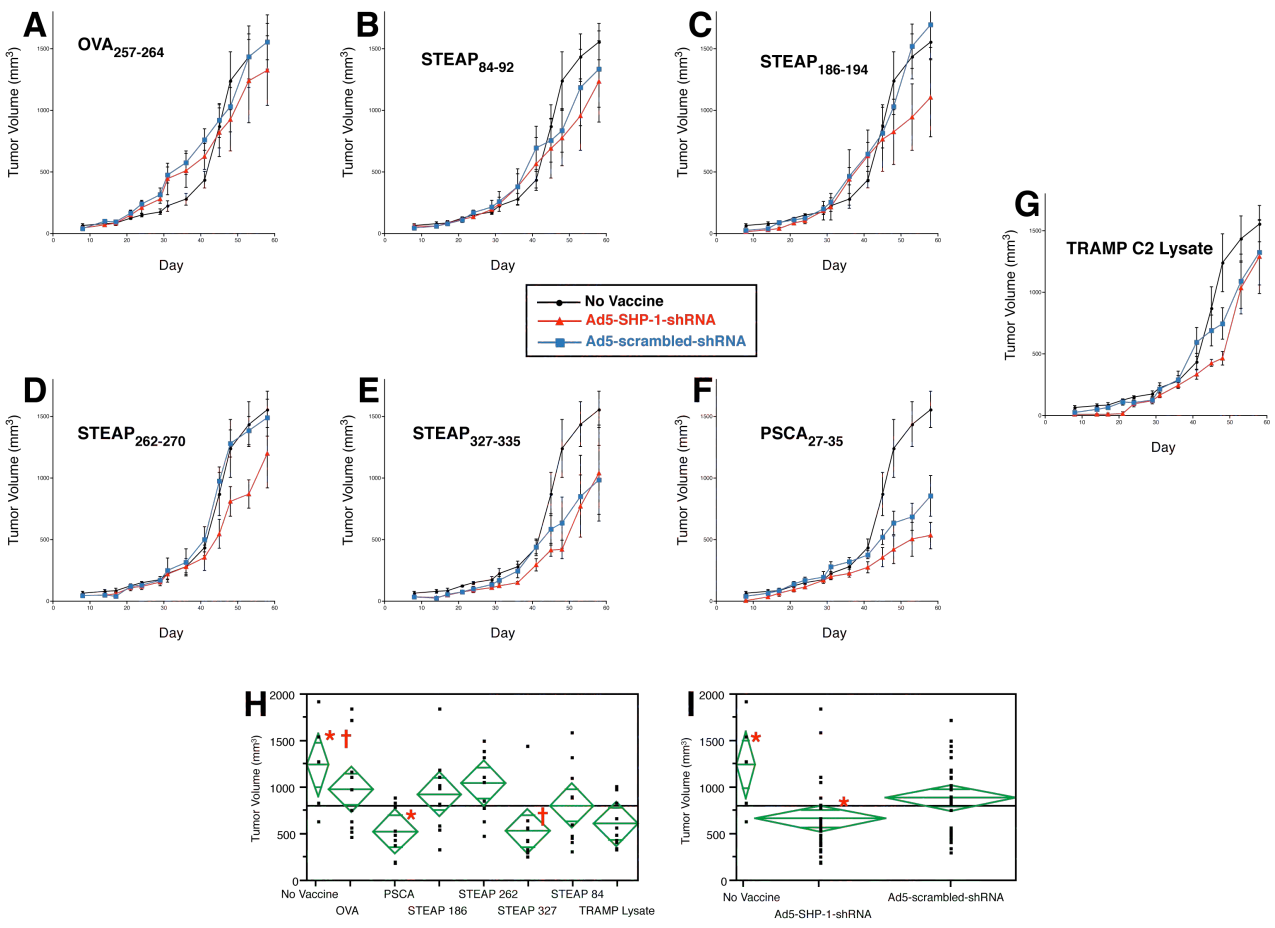

Figure 18. SHP-1 inhibition enhances DC vaccines against TRAMP C2 tumors. BMDCs were prepared as described above, transduced with either Ad5-SHP-1-shRNA or the control Ad5-scrambled-shRNA at 40,000 viral particles/ cell, and pulsed with one of the six peptides listed in Table 1 (OVA, an irrelavent epitope to TRAMP 2 tumors, served as a negative control peptide) or a lysate of TRAMP C2 cells as a positive control antigen. 6-8 week C57BL/6 mice, bearing TRAMP C2 tumors ($7x10^6$ cells injected s.c. on the dorsal flank three days prior to vaccination), were given a single i.p. vaccination with $2x10^6$ treated DCs or left untreated. Tumors were measured (length and width using calipers) every 2-4 days until termination of the experiment and tumor volume was estimated using the formula: Tumor volume (mm3) = x^2y x 0.5236, where x is the smaller tumor dimension, and y is the larger tumor dimension. A-G) TRAMP tumor growth curves for vaccines loaded with the indicated peptides. Error bars represent the standard error of the mean of 5 mice vaccinated in each treatment. These data are from one of 2 separate experiments each showing similar results. H-I) ANOVA analysis of peptide function and SHP-1 function. Data represent the averages 10 mice per peptide treatment and 5 mice in the no treatment control group for H) and 35 mice per viral transduction treatment and 5 mice in the no treatment control group for I). The green diamonds represent the mean and 95% confidence interval for each group from the ANOVA. The black line is the mean of means for the experiment and red symbols indicate significant differences ($p<0.05$ by Tukey-Kramer HSD multiple comparisons test).

p<0.005). Multiple comparisons testing showed that there was a significant decrease in tumor growth rate for mice treated with either $PSCA_{29-37}$ or $STEAP_{327-335}$ loaded DC vaccines compared to mice receiving no vaccination Tukey-Kramer HSD q*=3.13, p<0.05; Fig.13H). No significant differences were shown between untreated mice and other peptide vaccinations.

We next asked if vaccines transduced with Ad5-SHP-1-shRNA inhibited tumor growth compared to transduction with Ad5-scrambled-shRNA or no vaccine treatment, irrespective of what peptide was used in the vaccine. To determine if SHP-1 knock down had an effect on vaccine efficacy we pooled mice across all peptide treatments and performed an ANOVA and Tukey-Kramer HSD multiple comparisons test (Fig. 18I). Vaccines where SHP-1 was deficient showed significantly lower tumor volumes compared with those that were untreated. There were no significant differences in tumor volume between untreated mice and mice treated with Ad5-scrambled-shRNA vaccines (ANOVA: df=2, F=5.69, p<0.01; Tukey-Kramer HSD q*=2.93, p<0.05). We have repeated this experiment and have seen identical results for both SHP-1 and peptide effects in both experiments. Taken together these data demonstrate that SHP-1 inhibition significantly enhances DC vaccine efficacy against TRAMP prostate tumors *in vivo*. In addition, these data show that we have defined two new MHC class I tumor epitopes expressed in TRAMP C2 tumors *in vivo* that can be utilized for anti-tumor immunotherapy in this animal model.

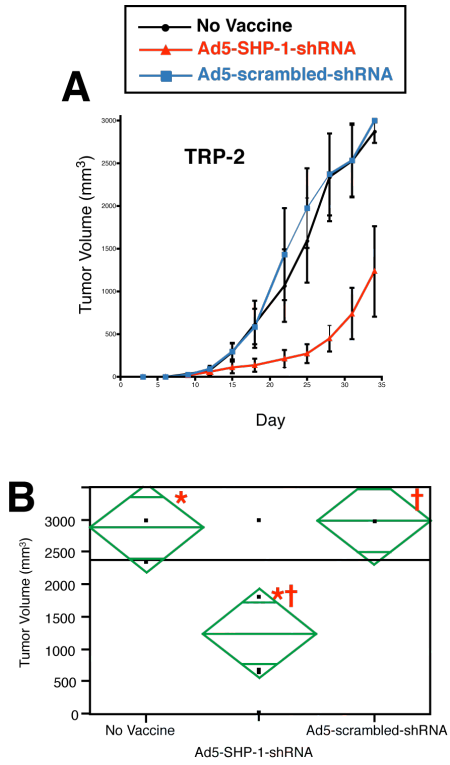

Figure 19. SHP-1 inhibition enhances DC vaccines against B16 tumors. BMDCs were prepared as described above, pulsed with the Trp-2 and then transduced with either Ad5-SHP-1 shRNA or the control Ad5-scrambled-shRNA virus at 40,000 viral particles/ cell. 6-8 week C57BL/6 mice, bearing B16 tumors ($5x10^5$ cells injected s.c. on the dorsal flank three days prior to vaccination), were given a single i.p. vaccination with $2x10^6$ treated DCs or left untreated. Tumors were measured every 2-4 days until termination of the experiment and tumor volume was calculated as described above. A) B16 tumor growth curves for vaccines loaded with Trp-2 peptide and transduced with viral vectors as indicated. Error bars represent the standard error of the mean of 5 mice vaccinated in each treatment. These data are from one of 2 separate experiments each showing similar results. B) ANOVA analysis SHP-1 function. The green diamonds represent the mean and 95% confidence interval for each group from the ANOVA. The black line is the mean of means for the experiment and red symbols indicate significant differences (p<0.05 by Tukey-Kramer HSD multiple comparisons test).

In an effort to further demonstrate that SHP-1 inhibition is effective in enhancing anti-cancer responses, against tumors other than the TRAMP C2 model, we performed vaccine experiments on mice bearing ectopic B16 melanoma tumors. The B16 melanoma, which is both aggressive and poorly immunogenic, expresses tyrosinase related protein-2 (Trp-2) of which peptide, SVYDFFVWL, is recognized by $CD8^+$ T cells in the context of $H\text{-}2K^b$. B16 represents a much more difficult tumor to treat than TRAMP and is considered the "gold standard" for ectopic tumor models in the C57BL/6 background. BMDCs were prepared as described above, pulsed with the Trp-2 and then transduced with either Ad5-SHP-1 shRNA or the control Ad5-scrambled-shRNA virus at 40,000 viral particles/ cell. 6-8 week C57BL/6 mice, bearing B16 tumors ($5x10^5$ cells injected s.c. on the dorsal flank three days prior to vaccination), were given a single i.p. vaccination with $2x10^6$ treated DCs or left untreated. Tumors were measured every 2-4 days until termination of the experiment and tumor volume was calculated as described above. Even with an inoculation 14 fold lower than that used for TRAMP C2 tumors, B16 tumors grow significantly faster and reach maximum acceptable size in 25-35 days in untreated mice (determined empirically from pilot experiments, data not shown). Mice vaccinated with DCs transduced with Ad5-SHP-1-shRNA showed markedly smaller mean tumor volumes (5 mice/treatment) beginning at day 18 than those vaccinated with Ad5-scrambled-shRNA transduced DCs or untreated mice (Fig. 19A). To determine if this difference was statistically significant, we performed an ANOVA and Tukey-Kramer HSD multiple comparisons test. Mice vaccinated with the SHP-1 deficient vaccine showed significantly lower mean tumor volume compared with either control group (ANOVA: df=2, F=9.82, p<0.005; Tukey-Kramer HSD q*=2.67, p<0.05; Fig. 19B). We have repeated this experiment and have seen the same SHP-1 effect in both experiments.

Further, we repeated the vaccine experiment in the B16 model comparing the shRNA and the dominant negative methods of SHP-1 inhibition. In this experiment BMDCs were transduced with either Ad5-SHP-1-shRNA or Ad5-DN-SHP-1 or their appropriate controls for adenoviral infection (Ad5-scrambled-shRNA for shRNA or Ad5-empty-CMV a virus expressing the empty vector into which DN-SHP-1 was cloned). 6-8 week C57BL/6 mice, bearing B16 tumors ($5x10^5$ cells injected s.c. on the dorsal flank three days prior to vaccination), were given a single i.p. vaccination with $2x10^6$ treated DCs or left untreated. Tumors were measured every 2-4 days until termination of the experiment and tumor volume was calculated as described above. Both SHP-1 inhibition treatment vaccine groups (shRNA or DN-SHP-1), slowed tumor growth compared to the control transduced DCs, untransduced DCs or no vaccine controls. Taken together, these data and those of the several tumor experiments described above, demonstrate that SHP-1 inhibition significantly enhances DC vaccine efficacy against both TRAMP and B16 tumors *in vivo*.

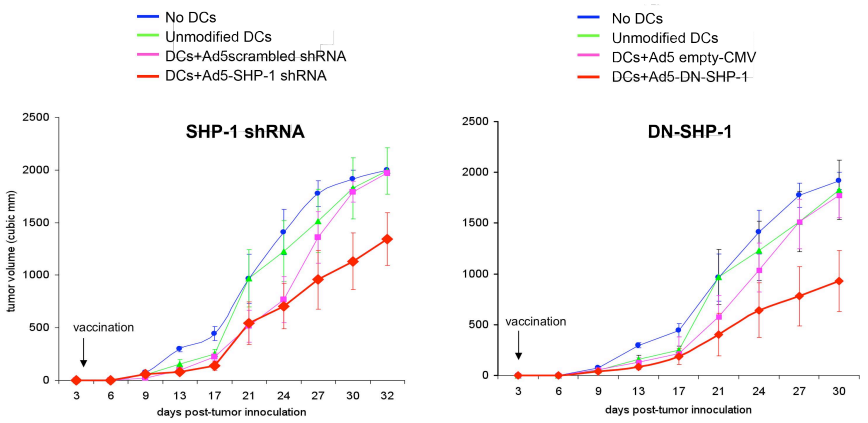

Figure 20. SHP-1 inhibition enhances DC vaccines against B16 tumors. BMDCs were prepared as described above, pulsed with the Trp-2 and then transduced with either Ad5-SHP-1 shRNA, the control Ad5-scrambled-shRNA, the Ad5-DN-SHP-1, or the control Ad5-empty-CMV virus at 40,000 viral particles/ cell or left untreated. 6-8 week C57BL/6 mice, bearing B16 tumors ($5x10^5$ cells injected s.c. on the dorsal flank three days prior to vaccination), were given a single i.p. vaccination with $2x10^6$ untreated DCs, or $2x10^6$ virally transduced DCs, or received no DC vaccine. Tumors were measured every 2-4 days until termination of the experiment and tumor volume was calculated as described above. Error bars represent the standard error of the mean of 6 mice vaccinated in each treatment.

KEY RESEARCH ACCOMPLISHMENTS

• Created 2 methods (shRNA and dominant negative SHP-1) for inhibiting SHP-1 function in DC.
• Generated high titer adenovirus expressing these inhibiting constructs for DC transduction.
• Demonstrated the efficacy of these constructs in modulating DC signaling through AP-1 and NFκB pathways.
• Demonstrated that SHP-1 inhibition in DC enhances their ability to migrate to CCR7 ligands.
• Demonstrated that SHP-1 inhibition in DC enhances their survival.
• Demonstrated that SHP-1 inhibition in DC enhances effector T cell function by upregulating Th1 skewing and down-regulating Treg induction.
• Defined novel peptide epitopes expressed by mouse TRAMP-C2 prostate tumors that are effective against tumors *in vivo*.
• Demonstrated that SHP-1 knockdown or dominant negative inhibition in DC vaccines reduces tumor growth in both B16 melanoma and TRAMP-C2 prostate tumor models.

REPORTABLE OUTCOMES

Abstracts

Dendritic cell anti tumor vaccine is enhanced by inhibiting the SH2-Domain containing phosphatase, SHP-1.
J.M. Levitt and I. R Ramachandran. Cancer immunology and immunotherapies Conference, National Cancer Institute, Bethesda, MD September 11-12, 2008

Dendritic cell prostate cancer vaccine is enhanced by inhibiting the SH2-Domain containing phosphatase,SHP-1.
J.M. Levitt and I. R Ramachandran. 7th World Basic Urological Research Congress meeting, Dublin, Ireland. September 26-29, 2007

The Role of SHP-1 in enhancing dendritic cell-based anti-tumor vaccines for prostate cancer.
A. Tewoldeberhan, I.R. Ramachandran, J.M. Levitt. Department of Defense, Innovative Minds in Prostate Cancer Today (IMPaCT) meeting, Atlanta, GA. September 5-8, 2007

Enhancing Dendritic Cell Tumor Vaccines by Inhibiting the SH2 Domain-Containing Phosphatase 1, SHP-1.
I.R. Ramachandran and J.M. Levitt. FASEB/American Association of Immunologists annual meeting Miami, FL. May 18-22, 2007

Patents

U.S. Patent Application No.: 12/122,146
Title: Inhibition of the SH2-domain containing protein tyrosine phosphatase, SHP-1, to enhance vaccines.
Inventors: Jonathan Levitt, Indu Ramachandran, Kevin Slawin
Filing Date: May 16, 2008

CONCLUSIONS

The results of the B16 and TRAMP tumor experiments (4 independent experiments in 2 different tumor models) clearly demonstrate that SHP-1 signaling in DCs constitutes a major inhibitory pathway, significant in its ability to down-regulate the initiation of antigen specific $CD8^+$ T cell responses *in vivo*. These tumor data are augmented by the mechanistic data indicating that SHP-1 inhibition, enhances DC activation signaling, survival, migration, and the ability of DCs to skew signaling towards a pro-inflammatory $CD4^+$ Th1 immune response while inhibiting Treg induction. The implication of these data in concert, is that SHP-1 signaling is a feasible protein to target in the design and implementation of DC-based vaccines against tumors and potentially against other infectious diseases.

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APPENDICES

None